Journal of Science and Technology ***55*** *(1A) (2017) 117-123*
DOI: 10.15625/2525-2518/55/1A/12388

IDENTIFICATION OF THE ENTOMOPATHOGENIC FUNGI SAMPLE DL0069 BY COMBINATION OF MORPHOLOGICAL AND MOLECULAR PHYLOGENETIC ANALYSES

Vu Tien Luyen[1], Lao Duc Thuan[2], Truong Binh Nguyen[3], Dinh Minh Hiep[4], Le Huyen Ai Thuy[2,*]

[1]*University of Science, Nguyen Van Cu Street, District 5, Ho Chi Minh City*
[2]*HCMC Open University, 97 Vo Van Tan Street, District 3, Ho Chi Minh City*
[3]*Da Lat University, 01 Phu Dong Thien Vuong Street, Da Lat City, Lam Dong Province*
[4]*Management Board of Agricultural Hi-Tech Park, Cu Chi District, Ho Chi Minh City*

[*]Email: *thuy.lha@ou.edu.vn*

Received: 30 October 2016; Accepted for publication: 30 May 2017

ABSTRACT

Species of *Simplicillium* are biological control agents against certain plant diseases caused by insects and nematodes due to their ability to parasite and kill the host. Recently, this anamorphic genus is classified under *Cordycipitaceae* as a monophyletic group apart from the type genus *Cordyceps*. In this current research, we reported the combination of morphological data and molecular phylogenies to identify an entomopathogenic fungal sample (DL0069) found in the mountainous regions of Langbian mountain, Lam Dong Province, Vietnam. Through formation of phialides and conidial chains, DL0069 was most likely a member of *Simplicillium* genus. From molecular phylogenetic analyses of a portion of the nuclear large ribosomal unit (nrLSU), it was confirmed that DL0069 was most closely related with *Simplicillium chinense*, a recently found *Simplicillium* species with a high potency as a biocontrol of nematodes parasiting plants.

Keywords: *Simplicillium*, entomopathogenic fungi, *nrLSU*, *Cordycipitaceae.*

1. INTRODUCTION

Since its first description, the genus *Simplicillium* Gams W. & Zare R. received few interest with only 6 new species described. Current member species include *S. lanosoniveum* (F.H Beyma) Zare R. & Gams W. (type species), *Simplicillium obclavatum* (W. Gams) Zare R. & Gams., *S. lamellicola* (F.E.V. Sm.) Zare R. & Gams W., *S. chinense* F. Liu & L. Cai, *S. subtropicum* Nonaka, Kaifuchi & Masuma, *S. minatense* Nonaka, Kaifuchi & Masuma, *S. cylindrosporum* Nonaka, Kaifuchi & Masuma, *S. aogashimaense* Nonaka, Kaifuchi & Masuma and *S. sympodiophorum* Nonaka, Kaifuchi & Masuma [1-3]. Representatives of this genus can be found in various sources including fungi, insects, nematodes, soils, human nails etc. [4].

Although species within *Simplicillium* are found to be entomopathogenic, they can also infect other fungi and on nematodes in different stages. The use of *Simplicillium* sp. as a biocontrol is limited due to its weaker virulence in comparison with *Beauveria bassiana* [4]. However, Le DQ et al. [5] have recently reported the antimicrobial activities of *Simplicillium lamellicola*, which may lead to the increase use of this genus as a biological control agent again in near future.

Despite the similarities between *Simplicillium* and *Lecanicillium*, *Simplicillium* sp. can be identified clearly from other related genera by combining morphological characteristics and phylogenetic information. Specifically, *Simplicillium* species are very similar to *Lecanicillium* in morphology except for the production of solitary phialides arising from aerial hyphae [1]. Phylogenetically, *Simplicillium* formed a monophyletic group apart from other clades including *Cordyceps* within *Cordycipitaceae* family [1, 6]. In this current research, we report the use of a portion of the nuclear ribosomal large subunit gene (*nrLSU*) to identify an entomopathogenic sample (DL0069) found in the mountainous regions of Langbian, Da Lat, Lam Dong, Vietnam.

2. MATERIALS AND METHODS

2.1. Sample isolation and morphological analysis

DL0069 was obtained from the fungi collection at Da Lat University as cultured mycelia in PGA medium. The sample was found on a fieldtrip to LangBian mountainous region. The sample was classified using macro- and micro- characteristics and the descriptions of Zare et al. [1], Liu & Cai [3] and Nonaka et al. [2].

2.2. DNA extraction, PCR and sequencing

DNA was isolated from the mycelia on PGA disks based on our previous protocol [7]. Shortly, mycelia was collected by a sterile stem and transfered into a tube containing lysis buffer. The mixture was incubated overnight at 65 ^{o}C and supernatant was collected after centrifugation. PCI (Phenol/Chloroform/Isoamylalcohol) solution was then added to the mixture. After centrifugation, the upper solution was collected for DNA precipitation by absolute ethanol. DNA concentration was identified by using OD260. The sample was kept in TE buffer at -20 ^{o}C.

PCR was conducted using LR0R and LR5 primers at binding temperature of 55 ^{o}C. PCR product was then analyzed using electrophoresis and sent to Nam Khoa Company for sequencing using the same pair of primer. DNA sequences were then proofread before phylogenetic analysis.

2.3. Molecular phylogenetic analysis

MEGA 7 software was used for phylogenetic analyses. Based on BLAST results and Liu & Cai (2012) publication, a database of 37 sequences (Table 1) was chosen for molecular phylogenetic analysis.

Phylogenetic reconstruction methods include Neighbor-Joining (NJ), Maximum Parsimony (MP) and Maximum Likelihood (ML) with 1000 bootstrap replicates. Before analysis, the database was subjected to model fitting to find the most suitable model of substitution.

Table 1. List of sequences used for molecular phylogenetics.

No	Sequence name	Accession number
1	*Akanthomyces arachnophilus*	EU369031
2	*Akanthomyces novoguineensis*	EU369032
3	*Ascopolyporus polychrous*	DQ118737
4	*Ascopolyporus polychrous*	AY886547
5	*Beaveria caledonica*	AF339520
6	*Bionectria epichloe*	DQ363259
7	*Bionectria orchroleuca*	AY489716
8	*Calonectria colombiana*	GQ280783
9	*Calonectria colombiana*	GQ280782
10	*Cordyceps militaris*	HM595906
11	*Engydontium album*	HM214541
12	*Engydontium album*	AF049167
13	*Gibellula* sp.	EU369037
14	*Hyperdermium bertonii*	AF242354
15	*Hypocrea virens*	AF399252
16	*Hypocrea viridescens*	HM535608
17	*Isaria farinose*	EF469080
18	*Isaria farinose*	DQ518773
19	*Lecanicillium lecanii*	EF464573
20	*Lecanicillium psalliotae*	AF049178
21	*Microhilum oncoperae*	AF339532
22	*Phytocordyceps ninchukispora*	EF468847
23	*Phytocordyceps ninchukispora*	EF468846
24	*Phytocordyceps ninchukispora*	FJ765239
25	*Pochonia gonioides*	AF339550
26	*Pochonia rubescens*	AF339566
27	*Simplicillium chinense*	KJ130983
28	*Simplicillium chinense*	JQ410321
29	*Simplicillium chinense*	JQ410322
30	*Simplicillium lamellicola*	NG_042381
31	*Simplicillium lamellicola*	AF339552
32	*Simplicillium lanosoniveum*	AF339553
33	*Simplicillium lanosoniveum*	KT878331
34	*Simplicillium lanosoniveum*	KT878334
35	*Simplicillium lanosoniveum*	HQ232006
36	*Simplicillium lanosoniveum*	AF339554
37	*Simplicillium obclavatum*	AF339517

3. RESULTS AND DISCUSSION

3.1. Morphological characteristics

DL0069 was found under the dry leaves of LangBian Mountain at the height of 1650 m with slim stromata, brown at the bottom and gray white in the middle with dark brown mature perithecia near the tip (Figure 1a).

Under light microscopy, perithecia with thickened wall formed in group and superficial from the base (Figure 1b). Ascus were formed in parallel inside perithecia. Upon maturation, the perithecial apex opened to relase the ascus. Ascospores were cyclindrical (Figure 1c).

After 3 days of inoculation, solitary phialides were observed (Figures 1e and f), which resembles the characteristics of *Simpicillium*. Conidia formed in chains with cylindrical shape (Figure 1d). From these data and the work of Liu & Cai [3], DL0069 is most likely *S. obclavatum* or *S. chinense.*

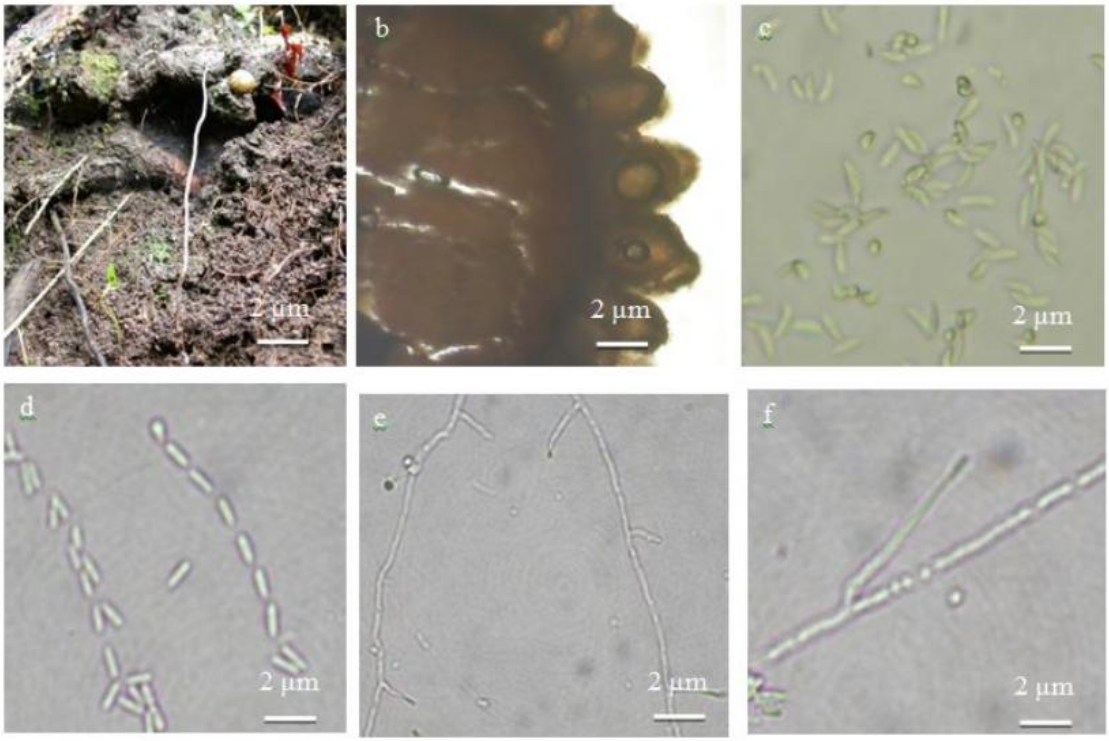

Figure 1. Macroscopic and mircoscopic characteristics of DL0069.
a. Stromata, b. Perithecia, c. Ascospores, d. Conidia, e-f. Phialides.

3.2. Phylogenetic analyses

The PCR product showed a band at ~900 bp (data not shown) and was sent for sequencing. After proofreading, the final sequence length was 723 bp and was incorporated into a database of 37 sequences formed by BLAST results and from the publication of Liu & Cai [3]. The dataset included representatives of *Cordycipitaceae* (29 sequences) including the type genus *Cordyceps* with type species *Cordyceps militaris* and other genera of the family including

Beaveria, *Simplicillium*, *Akanthomyces*, *Gibellula*, etc. Representatives of other family belonging to *Hypocreales* order were also presented including *Clavicipitaceae*, *Hypocreaceae*, *Nectriaceae* and *Bionectriaceae* (2 sequences/family).

Alignment was conducted on MEGA 7 using standard parameters. Ambiguous regions were removed and the final dataset was 741 bp long. The most fit model was the Tamura 3-parametr with Gamma distribution. Phylogenetic trees showed similar results between the three methods (Figure 2). Tree topologies were similar to that of Liu & Cai [3] despite the difference in dataset length. The difference between dataset lengths can be explained by the proofreading and alignment steps.

Species of *Cordycipitaceae* formed a monophyletic group with varied bootstrap values among the three tree construction methods and was moderately supported in Neighbor Joining tree while poorly supported in the other two methods. This phenomenon is similar to that of Liu & Cai [3] report. Using only Maximum Parsimony, the tree reconstructed by Lui & Cai [3] also showed a poor bootstrap support for *Cordycipitaceae* (under 50 %). This could be explained by the capacity of *nrLSU* to clarify the relationship between families of Hypocreales order, which can be overcome by using multigene phylogenies (Sung et al. [6]). *Cordycipitaceae* was also separated from other families of Hypocreales with similar bootstrap value compared to that of Liu & Cai [3]. Therefore, we concluded that the phylogenetic analyses were well designed and could be further use for identification of the fungi sample DL0069.

In all trees, *Simplicillium* formed a monophyletic group within *Cordycipitaceae* with bootstrap values 96/87/92 respectively for NJ, MP and ML methods. Specifically, in Maximum Parsimony tree, the *Simplicillium* clade was moderately supported. However, the bootstrap value in our research is higher than previous publication (77 %, Liu and Cai [3]). This could be explained by the number of taxa used for the molecular phylogenies. Liu & Cai [3] only used 6 sequences while our current database is comprised of 11 *Simplicillium* sequences. Moreover, DL0069 formed a monophyletic group within *S. chinense* (Figure 2) with high bootstrap values (100/99/98) and was similar to that of Liu & Cai [3].

Therefore, DL0069 is a specimen of *S. chinense*. *Simplicillium chinense* was firstly described by Liu & Cai [3] with long conidial chains containing longer and thinner condia compared to that of *S. obclavatum*, which marks the characteristics of the species. Recently, *S. chinense* was reported to be a potential agent against parasitic plant nematodes that can be used in combination with *B. bassiana* to control other diseases caused by insects and nematodes [8].

4. CONCLUSION

From morphological data, DL0069 was most likely a member of *Simplicillium* genus. Through molecular phylogenetic analysis, DL0069 was confirmed to be *Simplicillium chinense*, which have potential applications in agriculture as a nematode control agent. It is the first observation of this species in LangBian Mountain, Lam Dong, Viet Nam. However, in order to improve the identification process, other characteristics include the size and shape of conidial must be investigated for morphological comparisons and other nuclear regions such as the internal transcribed spacer (ITS) should also be explored.

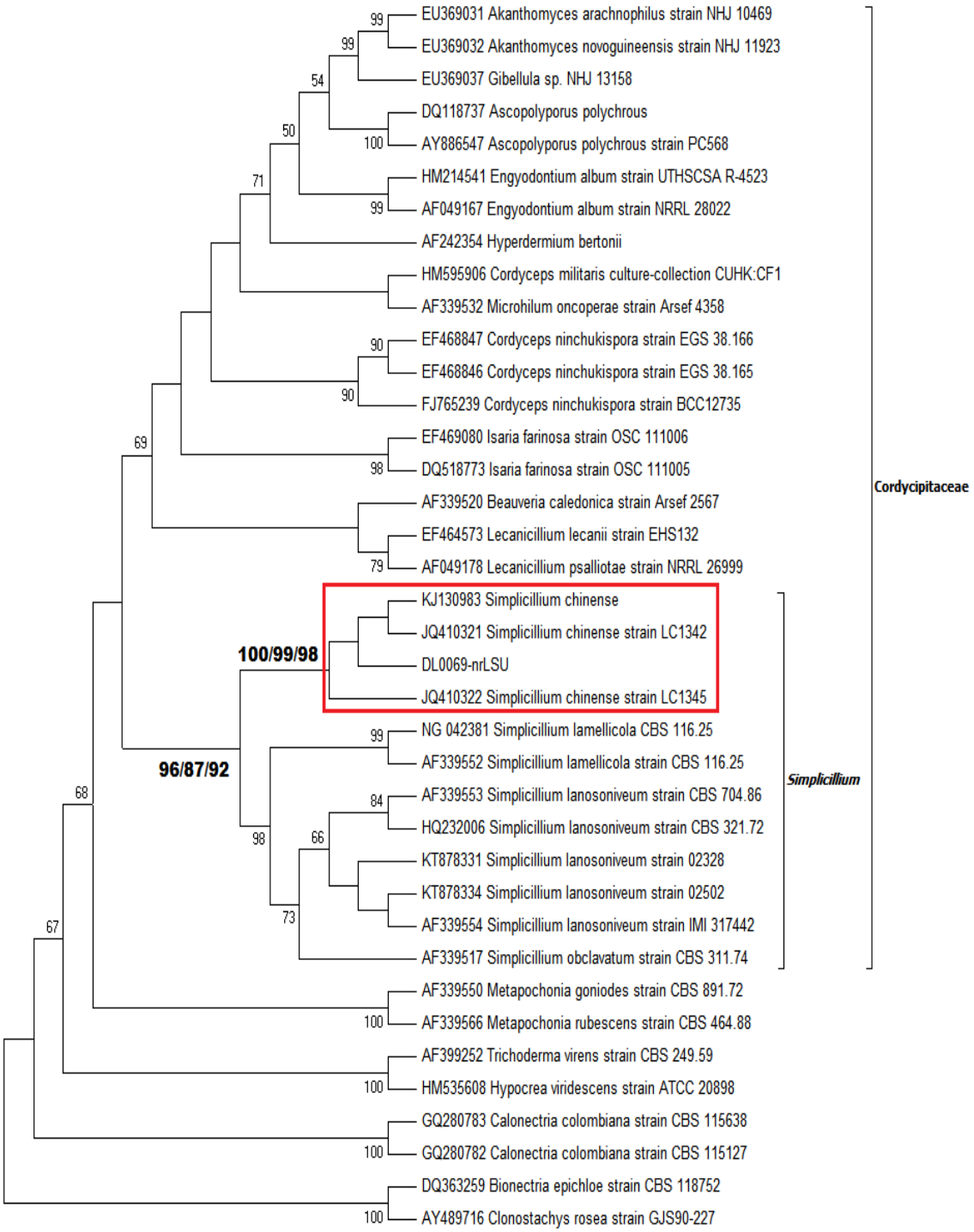

Figure 2. Phylogenetic analysis of DL0069 and related species by Maximum Parsimony with 1000 bootstrap replicates. One of the four most parsimonious trees is shown. The red rectangular is the *Simplicillium chinense* clade. Bootstrap values of the *S. chinense* and *Simplicillium* clades are presented with Neighbor-Joining, Maximum Parsimony, and Maximum Likelihood, respectively. Bootstrap values under 50 % is not presented in this tree.

Acknowledgement: This research was conducted on the support of the Ho Chi Minh City Department of Science and Technology and the Young Scientist Program 2014 – 2015 for Lao Duc Thuan (MSc).

REFERENCES

1. Zare R. and Gams W. - A revision of *Verticillium* section Prostrata IV. The genera *Lecancillium* and *Simplicillium* gen. nov., Nova Hedwigia **73** (2001) 1–50.
2. Nonaka K., Kaifuchi S., Ōmura S. and Masuma R. - Five new *Simplicillium* species (Cordycipitaceae) from soils in Tokyo, Japan, Mycoscience **54** (2013) 42–53.
3. Liu F. and Cai L. - Morphological and molecular characterization of a novel species of *Simplicillium* from China, Cryptogam Mycol **33** (2012) 137–144.
4. Lim S. Y., Lee S., Kong H. G., Lee J. - Entomopathogenicity of *Simplicillium lanosoniveum* isolated in Korea, Mycobiology **42** (4) (2014) 317–321.
5. Le D. Q., Shin T. S., Park M. S., Choi Y. H., Choi G. J., Jang K. S., Kim I. S. and Kim J. C. - Antimicrobial activities of novel mannosyl lipds isolated from the biocontrol fungus *Simplicillium lamellicola* BCP agains phytopathogenic bacteria, J Argi Food Chem **62** (15) (2014) 3363–70.
6. Sung G. H., Hywel-Jones N. L., Sung J. M., Luangsa-ard J. J., Shrestha B., Spatafora J. W. - Phylogenetic classification of *Cordyceps* and the Clavicipitaceous fungi, Studies in Mycology **57** (2007) 5–59.
7. Vu T. L., Trinh V. H., Trinh H. L., Nguyen T. B. D., Dinh M. H., Truong B. N, Lao D. T. - Discovery of entomopathogenic fungi *Cordyceps takaomontana* at LangBian Mountain, Lam Dong, Vietnam, The Journal of Science, Ho Chi Minh City Open University **1** (13) (2015) 14–20.
8. Zhao D., Liu B., Li L. Y., Zhu X. F., Wang Y. Y., Wang J. Q., Duan Y. X., Chen L. J. - *Simplicillium chinense*: a biological control agent against plant parasitic nematodes. Biocontrol Science and Technology **23** (8) (2013) 980–986.